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Development of a LC method for pharmaceutical quality control of the antimetastatic ruthenium complex NAMI-A

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Abstract

Imidazolium *trans*-tetrachloro(dimethylsulfoxide)imidazoleruthenium(III) (NAMI-A) is a novel ruthenium complex with selective activity against metastases currently in Phase I clinical trials in the Netherlands. Pharmaceutical quality control of NAMI-A drug substance and lyophilized product warranted the development of an assay for determination and quantification of NAMI-A and degradation products. A high performance liquid chromatography (HPLC) method was developed, consisting of a C18 column with 0.50 mM sodium dodecylsulfate in 3% methanol at pH 2.5 (acidified using trifluoromethanesulfonic acid) as the mobile phase and UV-detection at 358 nm. The HPLC method was proven to be linear, accurate and precise. Stress testing showed that degradation products were separated from the parent compound. By combining results of nuclear magnetic resonance (NMR) and HPLC experiments, one degradation product was identified as the mono-hydroxy species of NAMI-A. HPLC analysis with off-line detection of the eluate with flameless atomic absorption spectrometry (F-AAS) showed that under most conditions, all ruthenium-containing compounds show a peak in the HPLC chromatogram and that all ruthenium applied to the column is recovered quantitatively. For completely degraded solutions of NAMI-A some ruthenium is retained on the column. Suitability of the HPLC method for the pharmaceutical quality control of NAMI-A lyophilized product was demonstrated.

Keywords: NAMI-A; High performance liquid chromatography (HPLC); Ruthenium; Nuclear magnetic resonance (NMR); Flameless atomic absorption spectroscopy (F-AAS)

1. Introduction

Imidazolium *trans*-imidazole dimethylsulfoxide tetrachlororuthenate (III), ImH[*trans*-$RuCl_4$(DMSO)Im] (NAMI-A) Fig. 1 [1] is a novel ruthenium

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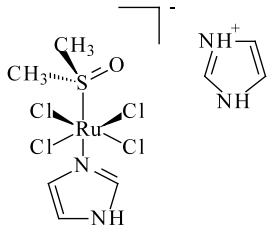

Fig. 1. Chemical structure of NAMI-A. Molecular weight = 458.18 g/mol. Molecular formula: $C_8H_{15}Cl_4N_4ORuS$.

anticancer agent. Preclinical pharmacological studies with NAMI-A showed selective activity against lung metastases of murine tumors [1–3] and low toxicity in mice and dogs [4–6]. Its action seems to be independent of the origin (type of primary tumor) and stage of growth of the metastases [2,7]. The precise mechanism of action, however, has yet to be proven, although recent evidence suggests an interaction with cell cycle regulation, inhibition of matrix metalloproteinases, and induction of antiangiogenesis [7–10].

Based on its promising activity and toxicity profile, NAMI-A is developed as a potential anticancer agent and has currently entered Phase I clinical trials. NAMI-A is pharmaceutically formulated as a lyophilized product for intravenous administration. The quality control of both drug substance and lyophilized product necessitated development of an assay for determination and quantification of NAMI-A and its potential degradation products.

This paper describes the development and validation of a stability-indicating reversed-phase high performance liquid chromatography (RP-HPLC) method with ultraviolet (UV) detection for pharmaceutical quality control of NAMI-A drug substance and lyophilized product. Furthermore, profiling of degradation products using deuterium nuclear magnetic resonance (^{2}H NMR) and ultraviolet/visible light (UV/VIS) spectrophotometry is reported. To test the quantitative recovery of ruthenium from the column and the ability of the HPLC system to detect all ruthenium-containing compounds, experiments are described where column eluates are analyzed for ruthenium with flameless atomic absorption spectrometry (F-AAS).

2. Experimental

2.1. Chemicals

NAMI-A was supplied by SIGEA Srl (Trieste, Italy). NAMI-A DMSO-d_6 (NAMI-A with a deuterated DMSO ligand) was kindly provided by Professor G. Mestroni (Department of Chemistry, University of Trieste, Italy). NAMI-A lyophilized product and distilled water were manufactured in-house (Department of Pharmacy and Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands).

Methanol (HPLC grade) was obtained from Biosolve Ltd. (Amsterdam, The Netherlands). Trifluoromethanesulfonic acid, sodium acetate trihydrate, acetic acid 96% (v/v), sodium dihydrogen phosphate dihydrate and hydrogen peroxide 30% (v/v) were purchased from Merck (Darmstadt, Germany) and sodium dodecylsulfate from Fluka Chimica GmbH (Buch, Switzerland). All reagents were of analytical grade and used without further purification.

2.2. UV/VIS spectrophotometry

UV/VIS spectra were recorded on a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia) equipped with a DELL personal computer and an Epson LX-400 printer. Spectra were recorded from 800 to 260 nm. Samples were prepared by dissolving NAMI-A in distilled water to a final concentration of 100 μg/ml.

2.3. High performance liquid chromatography

2.3.1. HPLC instrumentation and conditions

The HPLC system consisted of a model SP8800 ternary pump (Thermo Separation Products (TSP), Fremont, CA, USA), a model 996 photo diode array (PDA) detector (Waters, Milford, MA, USA) and a model SP8880 autosampler (TSP). Chromatograms were processed using Millenium® software (Waters). Separation was achieved using a μBondapak C18 column (Waters), protected with a C8 guard column (Security Guard, Phenomenex, Torrance, CA,

USA). The mobile phase consisted of 0.50 mM sodium dodecylsulfate in 3% methanol, acidified to pH 2.5 using trifluoromethanesulfonic acid (triflic acid). The flow rate was 0.5 ml/min and the system was operated at ambient temperature. The detection wavelength was 358 nm and on-line spectral analysis was carried out with the PDA system. The injection volume was 20 μl. A run time of 10 min was employed for the standard samples (calibration curve and quality control), and a run time of 30 min for stress testing samples to detect degradation products.

2.3.2. Sample preparation

2.3.2.1. NAMI-A drug substance. 500 μg/ml samples of NAMI-A drug substance were prepared by diluting 500 μl of a 1 mg/ml stock solution in distilled water with 500 μl mobile phase.

2.3.2.2. NAMI-A lyophilized product. NAMI-A 50 mg/vial lyophilized powder for intravenous use was dissolved and diluted to 50.0 ml using distilled water. Of this solution 500 μl was diluted with 500 μl mobile phase, resulting in a theoretical sample concentration of 500 μg/ml NAMI-A.

2.4. Validation procedure

The HPLC method was validated with respect to the following parameters: linearity, accuracy, precision and stability-indicating capability. Validation was carried out in compliance with ICH guidelines [11].

2.4.1. Linearity

A 1 mg/ml stock solution of NAMI-A in mobile phase was diluted with mobile phase to six concentration levels (100, 200, 300, 400, 500 and 600 μg/ml). Three separate runs were analyzed in duplicate. Least squares regression analysis of concentration, weighted by the reciprocal of the concentration squared [1/(concentration)^2] versus the NAMI-A peak area was applied. Linearity of each calibration curve was evaluated both by visual examination and the F-test for lack of fit.

2.4.2. Accuracy and precision

Accuracy, between-day and within-day precision were determined by analyzing samples at four concentration levels (100, 300, 500 and 600 μg/ml in mobile phase) in triplicate in three separate analytical runs. Samples were prepared from a separate stock solution of 1 mg/ml NAMI-A in mobile phase. Accuracy was measured as the percentage deviation from the nominal concentration. Between-day and within-day precision were determined by performing one-way analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable. From ANOVA analysis, the day mean square (DayMS), error mean square (ErrMS) and grand mean (GM) were obtained. Between-day and within-day precisions were calculated using Eqs. (1) and (2), where n is the number of replicates.

$$\text{Between-day precision (\%)} = 100\times\sqrt{\text{ErrMS}/\text{GM}} \quad (1)$$

$$\text{Within-day precision (\%)} = 100\times\sqrt{((\text{DayMS}-\text{ErrMS})/n)/\text{GM}} \quad (2)$$

2.4.3. Stability-indicating capability

The capacity of the HPLC method to determine the presence of degradation products was tested by subjecting NAMI-A to stress conditions (acid, alkaline, neutral, heat, oxidation, and high intensity light). In all cases, 1 mg/ml solutions of NAMI-A were prepared and stored at room temperature in the dark (except for the solutions exposed to heat and high intensity light). For the acidic conditions, 0.1 M acetate buffer at pH 3 was prepared. Neutral and alkaline conditions were created by adjusting the pH of a 0.1 M phosphate buffer to pH 7 and 10, respectively. Throughout the experiments, the pH of the solutions was monitored and adjusted as necessary. A solution in distilled water was exposed to heat in a stove at 50 °C (Heraeus, Hanau, Germany) and to UV/VIS light in the range of 320–800 nm at an intensity of 765 W/m^2. To this purpose, a Suntest CPS$^+$ instrument with xenon lamp and glass filters with a cut-off at 320 nm (Atlas Materials Testing Technology LLC, Chicago, IL, USA) was used.

Oxidation of NAMI-A took place in 15% hydrogen peroxide. Each solution prepared for stress testing was sampled at different time points, depending on the rate of degradation. At selected time points, 500 μl samples were taken, diluted with 500 μl mobile phase and 20 μl of the resulting solution was injected into the HPLC system.

2.5. Profiling of degradation products

2.5.1. NMR spectroscopy

^{2}H NMR spectra were recorded using a Bruker AC 200 spectrometer at 30.7 MHz. NAMI-A DMSO-d_6 samples were dissolved in 0.15 M aqueous phosphate buffer (pH 3.5 and pH 7.4) at a concentration of 25 mM (11.45 mg/ml) and immediately analyzed. Spectra were stored at regular time intervals and each spectrum was measured with 128 scans.

The NMR experiments were duplicated using HPLC under identical storage conditions. Samples (45 μl) were taken every 30 min for 6 h, diluted with 955 μl mobile phase and injected into the HPLC system for the solutions at pH 7.4. For the solutions at pH 3.5, samples were taken immediately and every 2 h until 24 h after preparation.

2.5.2. F-AAS

Ruthenium concentrations were measured using a SpectrA-A 30/40 Zeeman Graphite Furnace Atomic Absorption Spectrometer (Varian, Techtron Pty Ltd, Victoria, Australia), using a procedure described earlier [12].

Solutions containing 25 mM NAMI-A in 0.15 M phosphate buffer at pH 3.5 and 7.4 were prepared. For both solutions, 85 μl samples were taken immediately after preparation, diluted with 915 μl mobile phase and injected into the HPLC system. Subsequently, the solutions were stored at room temperature, protected from light, and the next sample was taken after 24 h for the solution at pH 7.4 and after 72 h for the solution at pH 3.5. The chromatograms were recorded and fractions from the outlet of the PDA detector were collected every 30 s, resulting in 250 μl fractions. The ruthenium concentration in each fraction was measured using F-AAS. Furthermore, the total ruthenium concentration in each test solution was measured at the same time points as the fractions were collected.

3. Results and discussion

3.1. UV/VIS spectrophotometry

NAMI-A has a UV/VIS spectrum showing one major absorption band with a maximum at 390 nm. During storage of a 100 μg/ml solution of NAMI-A in distilled water at room temperature (+20–25 °C) and protected from light, its UV/VIS spectrum changes due to degradation as depicted in Fig. 2. An isosbestic point is observed at 358 nm. Similar changes in the UV/VIS spectrum take place when NAMI-A is dissolved in mobile phase.

3.2. High performance liquid chromatography development

Like cisplatin, NAMI-A consists of a metal with several ligands, including more than one chloride ion. Even though cisplatin forms a square planar structure and NAMI-A an octahedron and different types of ligands are present, NAMI-A has been shown to undergo similar substitution reactions of chloride by water as cisplatin [13,14]. Cisplatin is known to form mono- and di-aqua products, especially when stored in chloride-free solutions. With respect to this degradation pathway, it was necessary to develop an HPLC method capable of discerning the different hydration stages of NAMI-A. El-Khateeb et al. [15] investigated several HPLC-UV methods for the analysis of cisplatin. They found that some commonly used components of mobile phases such as acetonitrile and several carboxylic acids used for pH adjustment interact with the aqua complexes of cisplatin, making it impossible to properly analyze the degradation products. They developed an HPLC method utilizing a mobile phase consisting of 0.50 mM sodium dodecylsulfate (added as anionic hydrophobic ion-pairing agent) in 3% methanol, its pH adjusted to pH 2.5 using trifluoromethanesulfonic acid. In combination with this mobile phase, a μBondapak C18 column at 37 °C, flow of 0.5 ml/min and UV-detection at 305 nm provided

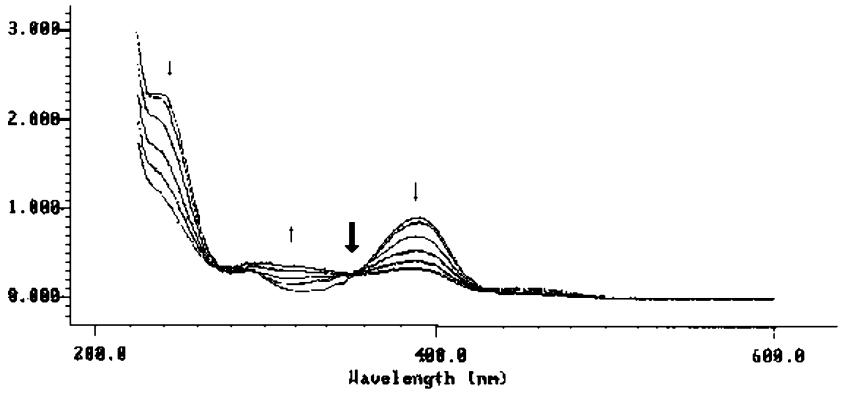

Fig. 2. Change in UV/VIS spectrum of a solution of 100 μg/ml NAMI-A in distilled water in time from $t = 0$ to 96 h (stored at 20–25 °C in the dark). The directions of the small arrows indicate an increase or decrease of absorption upon degradation; the large arrow indicates the isosbestic point at 358 nm.

the optimal system for separation of cisplatin from its aqua-complexes. This HPLC method was used as a starting point for the development of a method for the analysis of NAMI-A. Influence of column temperature (20–37 °C) on the separation of NAMI-A and its degradation products was negligible. UV-detection at 358 nm was selected, as this was the isosbestic point of NAMI-A degradation in distilled water and mobile phase (Fig. 2). The total peak area at 358 nm should remain unchanged during the course of degradation if all products show UV-absorption and elute from the column.

Injection of reference NAMI-A into this HPLC system resulted in a single peak in the chromatogram at a retention time of approximately 4 min, the identity being confirmed as NAMI-A by its UV/VIS spectrum. The absence of secondary peaks indicates negligible decomposition of NAMI-A during the chromatographic process.

3.3. Validation of the HPLC-UV method

3.3.1. Linearity

All calibration curves proved linear in the concentration range of 100–600 μg/ml, as confirmed by the F-test for lack of fit ($\alpha = 0.05$). For all three calibration curves correlation coefficients > 0.998 were calculated using least squares regression analysis. Table 1 shows the nominal and observed concentrations for the different concentration levels for the six analytical runs. The deviation from the nominal concentration was less than 2% for all concentration levels.

3.3.2. Accuracy and precision

The between- and within-day precisions were less than 5% and the accuracy within 3.1% (Table 2). The limit of detection of the method (the concentration NAMI-A producing a signal-to-noise ratio of 3) was 0.5 μg/ml.

3.3.3. Stability-indicating capability

Fig. 3 shows the percentage NAMI-A remaining with respect to the initial concentration at several time points under the different stress conditions. Table 3 shows the relative retention times, maxima of the UV/VIS spectra of the degradation peaks formed under these stress conditions, and the extent to which they were formed.

Clearly, the rate of degradation of NAMI-A is pH-dependent. NAMI-A is most stable in acidic solutions and degrades extremely fast in basic solutions, with an undetectable amount of NAMI-A remaining in the solution at pH 10 after 15 min. NAMI-A in solutions exposed to high intensity light, heat, and oxidation degrades slower than in solution at pH 10 but faster than in solution at pH 7 (Fig. 3). Like the acidic solution, the solution in hydrogen peroxide has a pH of approximately 3, but degrades more rapidly. Thus, oxidation catalyzes the degradation reaction or

Table 1
Validation results of the NAMI-A calibration curve samples ($n = 6$)

Nominal concentration (μg/ml)	Measured concentration (μg/ml) ± SD	RSD (%)	Accuracy (%)
100.41	99.8 ± 4.3	4.3	99.4
200.81	202.5 ± 7.6	3.7	100.9
301.22	306.9 ± 4.8	1.6	101.9
401.62	401.7 ± 12.5	3.1	100.0
502.03	497.8 ± 11.0	2.2	99.2
602.44	597.2 ± 12.1	2.0	99.1

causes a different type of degradation reaction to occur, possibly oxidation of ruthenium (III). If unadjusted in time (as was the case for the solutions exposed to light, heat, and oxidation), the pH of all NAMI-A solutions decreases to a value of approximately 3.5, indicating the formation of free protons as a result of deprotonation of the aqua complexes, leading to formation of ruthenium (III) hydroxides [13,14].

One degradation product (DP 1) is formed under all investigated conditions. It has a relative retention time between 1.63 and 1.89 and maxima in its UV/VIS spectrum at 360 and 420 nm. It is the main degradation product formed under all conditions, except oxidation and heat. Another degradation product (DP 2) appears under multiple conditions, all of acidic pH; it has a relative retention time between 1.40 and 1.51 and maxima in its UV/VIS spectrum at 326 and 374 nm. It can be speculated that DP 1, which is formed under all investigated conditions is a hydroxide of NAMI-A. This hydroxide would be formed by substituting one chloride ligand by a water molecule, which is then deprotonated, causing the solutions to become acidic if the pH is unadjusted, as was the case for the solutions exposed to heat, light, and oxidation. In alkaline media, a direct substitution of chloride by hydroxide can be expected. In acidic solution, DP 2 also appears, which may be a higher aquated complex, or a DMSO-free degradation product. Other degradation products appear to be formed only under influence of specific conditions, although exposure to heat and high intensity light show several identical degradation products. For other stress conditions, several peaks have very similar UV/VIS spectra to DP 2, yet are distinctly separated from it.

Under most stress conditions, the peak in the chromatogram attributed to NAMI-A (retention time approximately 4 min) retains the same UV/VIS spectrum during the course of degradation, which indicates that no degradation product with different spectroscopic properties than NAMI-A co-elutes with it. However, under alkaline conditions, when all NAMI-A has degraded (after approximately 15 min), a small peak with a different UV/VIS spectrum than NAMI-A emerges in the same position (relative retention time 1.00, maximum in its UV/VIS spectrum at 350 nm). This peak is most likely a degradation product typically formed under alkaline conditions and not an impurity, as it is not observed when all

Table 2
Validation results of the NAMI-A precision measurements

Nominal concentration (μg/ml)	Within-day precision (%)	Between-day precision (%)	Accuracy (%)
98.46	2.7	1.4	99.6
295.37	4.8	4.8	102.1
492.28	3.4	4.9	98.9
590.73	2.2	4.0	96.9

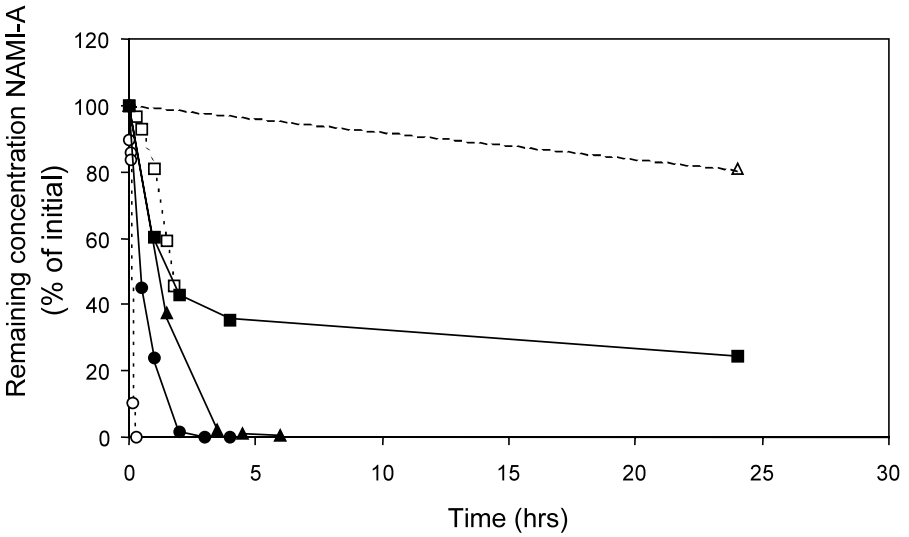

Fig. 3. Degradation of NAMI-A under different stress conditions (△ = acid; □ = neutral ○ = alkaline; ▲ = heat; ■ = oxidation; ● = light of high intensity).

NAMI-A in the solutions exposed to light of high intensity or heat has degraded.

Some variation of relative retention times is observed in the chromatographic analyses (see also Table 3). The absolute retention time of NAMI-A lengthened as the column was in use for longer periods of time, while the absolute retention time of the degradation products did not change to a large extent, decreasing the relative retention time with aging of the column.

The solutions in buffers (acidic, neutral and alkaline) retain their total peak area at the detection wavelength of 358 nm, but not the solutions exposed to light, heat, and oxidation, which show a decrease in total peak area in time. In these unbuffered solutions, a general loss in UV/VIS absorbance was also noted for completely degraded solutions, a phenomenon accompanied by a gradual darkening of the solution, indicating the formation of poly-oxo species [13,14].

Further studies were conducted to identify degradation products using ^{2}H NMR techniques. F-AAS experiments were performed to determine whether all ruthenium that was applied to the column was recovered and to investigate whether ruthenium-containing peaks could be discerned that were not visible in the HPLC chromatograms.

3.4. Degradation product profiling

3.4.1. ^{2}H NMR spectroscopy

^{2}H NMR studies were performed using 25 mM NAMI-A-DMSO-d_6 solutions, as lower concentrations (such as used during stress testing) did not lead to detectable levels of degradation products. Neutral (pH 7.4) and acidic (pH 3.5) solutions were selected as degradation media.

The NMR experiments in combination with UV/VIS analysis led to identification of several degradation products of NAMI-A and proposition of a degradation pathway as depicted in Fig. 4 [13,14]. The deuterated DMSO group is pivotal in the identification of some degradation products and (by the appearance of free DMSO) inferring the presence of others. The parent compound gives rise to a pH-independent, single resonance at −15 ppm. At neutral conditions, a second peak is identified at −11 ppm, which is likely to correspond to the mono-hydroxy complex derived from the replacement of one chloride ligand by hydroxide (compound B in Fig. 4). The resonance of this compound is pH-dependent and, by lowering the pH, moves towards lower ppm values, until at pH 3.5 it overlaps the NAMI-A parent compound (compound A) at −15 ppm. The pH dependence is probably a result of the protonation–deprotona-

Table 3
Degradation products formed under different stress conditions (DP 1 = degradation product 1, DP 2 = degradation product 2)

Conditions	Relative retention time	Absorption maxima (λ in nm)	% of total peak area of degradation product at end of experiment
Acidic	1.00 (NAMI-A)	288; 390; 452	85.1
	1.43 (DP2)	326; 374	1.4
	1.51	304	5.7
	1.72 (DP 1)	360; 420	6.6
	1.92	308	1.4
Alkaline	1.00	350	0.4
	1.54	365; 427	0.2
	1.75 (DP 1)	360; 420	99.4
Neutral	1.00 (NAMI-A)	288; 390; 452	31.3
	1.45	365; 427	0.1
	1.64 (DP 1)	360; 420	68.6
Oxidation	1.00 (NAMI-A)	288; 390; 452	46.2
	1.42 (DP 2)	326; 374	5.8
	1.48	368; 430	40.1
	1.64	365; 427	1.9
	1.70 (DP 1)	360; 420	5.9
Heat	1.00 (NAMI-A)	288; 390; 452	3.7
	1.40 (DP 2)	326; 374	18.5
	1.63 (DP 1)	360; 420	7.4
	4.84	318	25.7
	5.31	292; 355	44.5
High intensity light	1.51 (DP 2)	326; 374	23.7
	1.59	325; 371	2.4
	1.89 (DP 1)	360; 420	60.6
	3.04	318	4.8
	4.29	292; 355	8.5

tion equilibrium of the hydroxide ligand, which under acidic conditions forms a water molecule. Compounds D, E and F cannot be directly detected using ^{2}H NMR, but their presence can be inferred from the quantity of free deuterated DMSO.

Fig. 5A depicts the results obtained for the NMR degradation studies at pH 7.4. It is shown that NAMI-A (compound A in Fig. 4) is completely degraded within 2 h. The mono-hydroxy species of NAMI-A (compound B in Fig. 4), is formed and disappears again, due to consecutive degradation. Another compound is detected when almost all NAMI-A has disappeared and the mono-hydroxy species is at its maximum concentration. This compound is tentatively identified as the di-hydroxy species (compound C in Fig. 4). The relative concentration of free DMSO increases in time and reaches a plateau after 4 h, which indicates formation of DMSO-free degradation products (compounds D, E and F in Fig. 4).

Fig. 6 depicts the results of the NMR study performed at pH 3.5. An increase in free DMSO concentration is noted, which indicates formation of DMSO-free degradation products. Unfortunately, no quantitative distinction between the parent compound (compound A) and its mono-aqua species (compound B) is possible at acidic pH. The mono-aqua species appears as a shoulder in the NAMI-A peak, indicating its presence, but making quantification impossible. This shoulder appears after approximately 8 h. As free DMSO is detected immediately after dissolution, it can be deduced that at acidic pH, NAMI-A degrades primarily by hydrolysis of the DMSO ligand and chlorine substitution occurs only at a low rate.

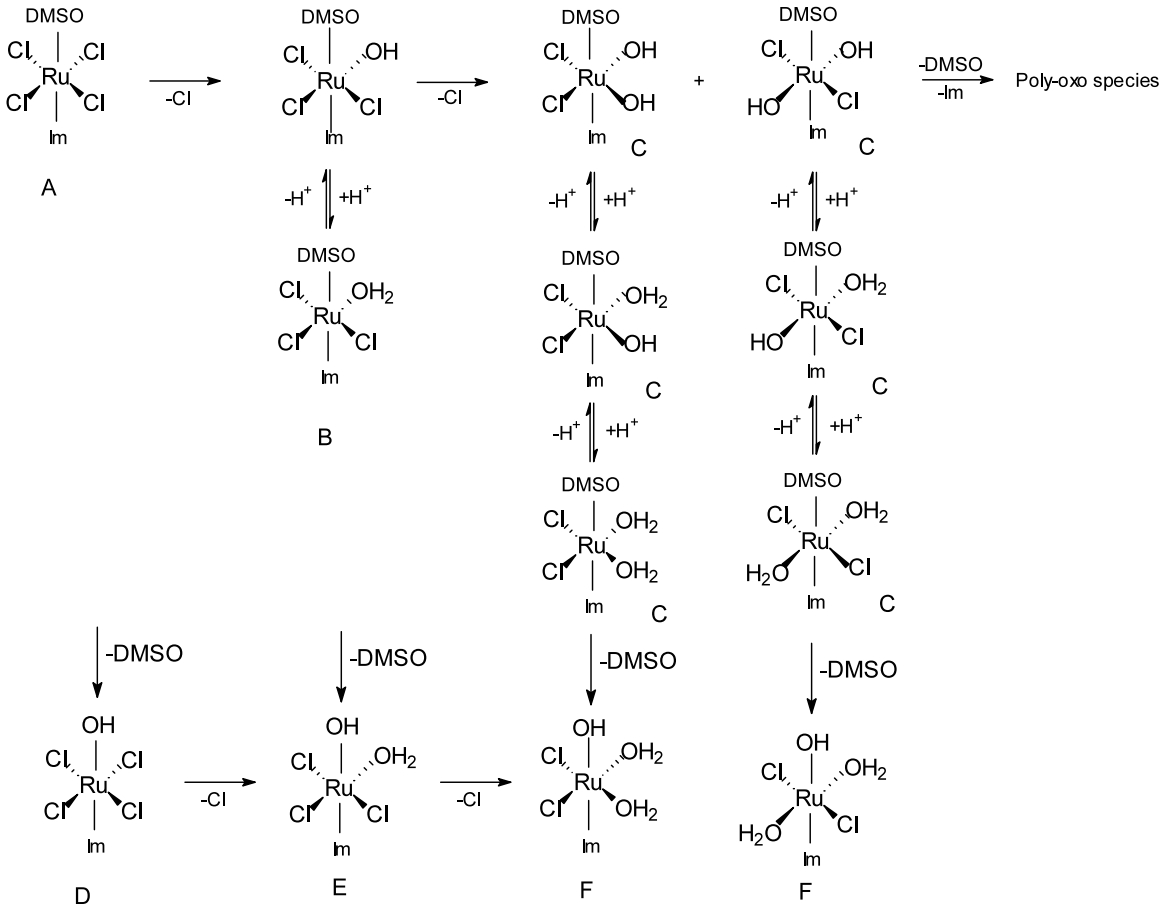

Fig. 4. Proposed degradation scheme of NAMI-A.

This is different from NAMI-A degradation at neutral pH, where chlorine substitution appears to play a more prominent role than DMSO hydrolysis.

3.4.2. Duplication of the NMR experiment with HPLC

The NMR experiments were duplicated using HPLC analysis in order to identify the peaks generated during degradation of NAMI-A in phosphate buffer at pH 3.5 and 7.4.

Fig. 5B depicts the results obtained for the HPLC degradation studies at pH 7.4. The areas of the observed peaks in the HPLC chromatogram plotted against time show a similar profile as the NMR plot. Immediately after preparation, the solution already contains a degradation product at a relative retention time of 1.5 and maxima in its UV/VIS spectrum at 360 and 420 nm (DP 1, Table 3). This peak grows in time, then decreases, in the same manner, but at a slightly slower rate as found for the mono-hydroxy species in the NMR experiments. This might be due to the slightly lower temperature (21 °C instead of 25 °C) at which this experiment was carried out. Based on these results, DP 1 was identified as the mono-hydroxy species of NAMI-A (compound B, Fig. 4). After 3 h at pH 7.4, when NAMI-A is undetectable, another degradation peak is formed in the HPLC chromatograms with a relative retention time of 1.0 and a maximum in its UV/VIS spectrum at 350 nm. This peak may be the degradation product which could not be assigned by NMR (tentatively the di-hydroxy species of NAMI-A: compound C in

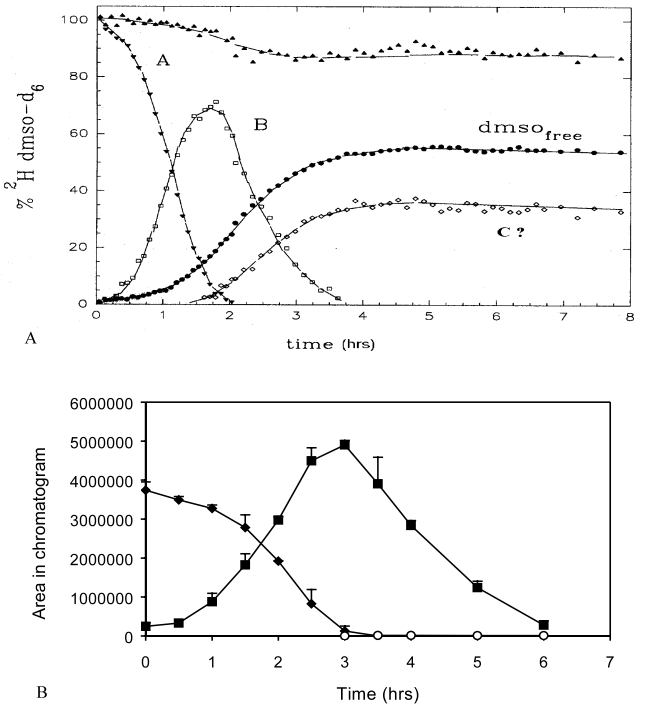

Fig. 5. A. NMR degradation study of NAMI-A in solution at pH 7.4 (▼ = NAMI-A (compound A), □ = compound B (mono-hydroxy/aqua species), ◇ = not identified with certainty, but likely compound C (di-hydroxy/aqua species), ▲ = total DMSO, ● = free DMSO). B. HPLC degradation study of NAMI-A in solution at pH 7.4 (◆ = NAMI-A, ■ = DP 1, ○ = peak with maximum in its UV/VIS spectrum at 350 nm).

Fig. 4), or a different degradation product, which is formed upon complete degradation of NAMI-A in alkaline solutions.

The HPLC study performed at pH 3.5 shows the slow formation of the mono-hydroxy species of NAMI-A (DP 1, relative retention time 1.5, UV/VIS maxima at 360 and 420 nm). Furthermore, a second degradation product (DP 2, Table 3) is formed. It has a relative retention time of 1.3 and maxima in its UV/VIS spectrum of 326 and 374

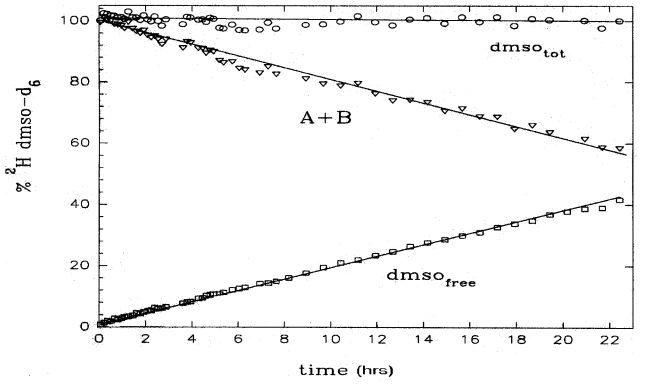

Fig. 6. NMR degradation study of NAMI-A in solution at pH 3.5 (▽ = NAMI-A and its mono-aqua/hydroxy species, □ = free DMSO, ○ = total DMSO).

nm and was also observed in all solutions at acidic pH during stress testing. NMR analysis showed that at acidic pH, NAMI-A degrades mainly by DMSO hydrolysis, thus DP 2 may be any one of the DMSO-free degradation products depicted in Fig. 4. This is apparently confirmed by the fact that when DMSO is added to an acidic solution, DP 2 is formed to a lesser extent. Thus, as hydrolysis of the DMSO group is likely prevented by addition of free DMSO, addition of DMSO would lead to less formation of the DMSO-free complexes.

3.4.3. General discussion of NAMI-A degradation

NAMI-A is a ruthenium(III) [Ru(III)] complex in an octahedral configuration and has rather complex chemistry. Ru(III) forms complexes in which the ligands are sensitive to exchange reactions. In aqueous solution the first chloride ion in ruthenium hexachloride, $[Ru(III)Cl_6]^{3-}$, is exchanged within a few seconds by a water molecule, while the last chloride ion in $[Ru(III)Cl(H_2O)_5]^{2+}$ to be replaced by a water molecule takes more than 1 year [16]. In this study, UV/VIS and NMR methods were used to obtain relevant insight into the behavior of NAMI-A in aqueous solution, especially with respect to its stability. The interpretation of the results obtained is of a rather speculative nature. No attention has been paid to the imidazole ligand, because neither the HPLC nor the UV/VIS method gives information about this ligand, although there are several unidentified peaks in the HPLC chromatograms. It can be argued that imidazole (Im), with a pK_a of 6.9, will be protonated in solutions of acidic pH, resulting in repulsion of like forces between Ru^{3+} and ImH^+, weakening the bond to a large extent. Therefore, it should not be surprising if Im is the most easily exchanged ligand under acidic conditions. However, it has been shown by NMR analysis that the imidazole ligand is not readily exchanged [14]. It seems likely that DMSO is hydrolyzed in preference to imidazole, as an increase in free DMSO is observed in NMR studies. In alkaline and neutral medium, the hydroxy-ion is the ligand of choice for exchange and it appears that the chloride ligands are preferentially substituted. Further investigation into the degradation mechanism of NAMI-A is ongoing. For the moment, the scheme as proposed in Fig. 4 remains the most logical degradation pathway.

3.5. F-AAS

Fig. 7 shows the HPLC chromatograms for a solution of NAMI-A in phosphate buffer at pH 7.4 immediately after preparation and after 24 h storage at room temperature in the dark and their corresponding F-AAS ruthenium concentration profiles. Fig. 8 shows the results for a solution at pH 3.5 immediately after preparation and after 72 h storage. The ruthenium profiles obtained by F-AAS for both solutions at $t = 0$ and for pH 3.5 at $t = 72$ h match their HPLC chromatograms. The total amount of ruthenium measured in the combined fractions was equal to the ruthenium content of the sample taken directly from the solution under investigation, indicating that the amount of ruthenium injected into the HPLC is completely recovered.

The solution at pH 7.4 after 24 h storage shows deviating results. This test solution contains approximately 43% of the initial ruthenium concentration. The loss of ruthenium is explained by the formation of a black precipitate in this solution. Visual precipitation starts after 6 h of storage at pH 7.4. This precipitate may contain insoluble ruthenium hydroxides, oxides, or the poly-oxo species formed upon further degradation (Fig. 4). Its formation was not observed in the neutral solution during stress testing, as the stress testing solution was measured for 2 h.

Along with loss of ruthenium due to precipitation, it appears that ruthenium is retained on the column. The total amount of ruthenium recovered from the collected fractions after injection onto the HPLC column contained 21% of the ruthenium present in the test solution at this time point. Furthermore, a large ruthenium peak is observed in the F-AAS ruthenium profile at a retention time of 9 min, which is observed in the HPLC chromatogram not as a peak, but as a deflection from baseline. This deflection, however, has a UV/VIS spectrum with a maximum absorption in its UV/VIS spectrum at 318 nm, indicating the

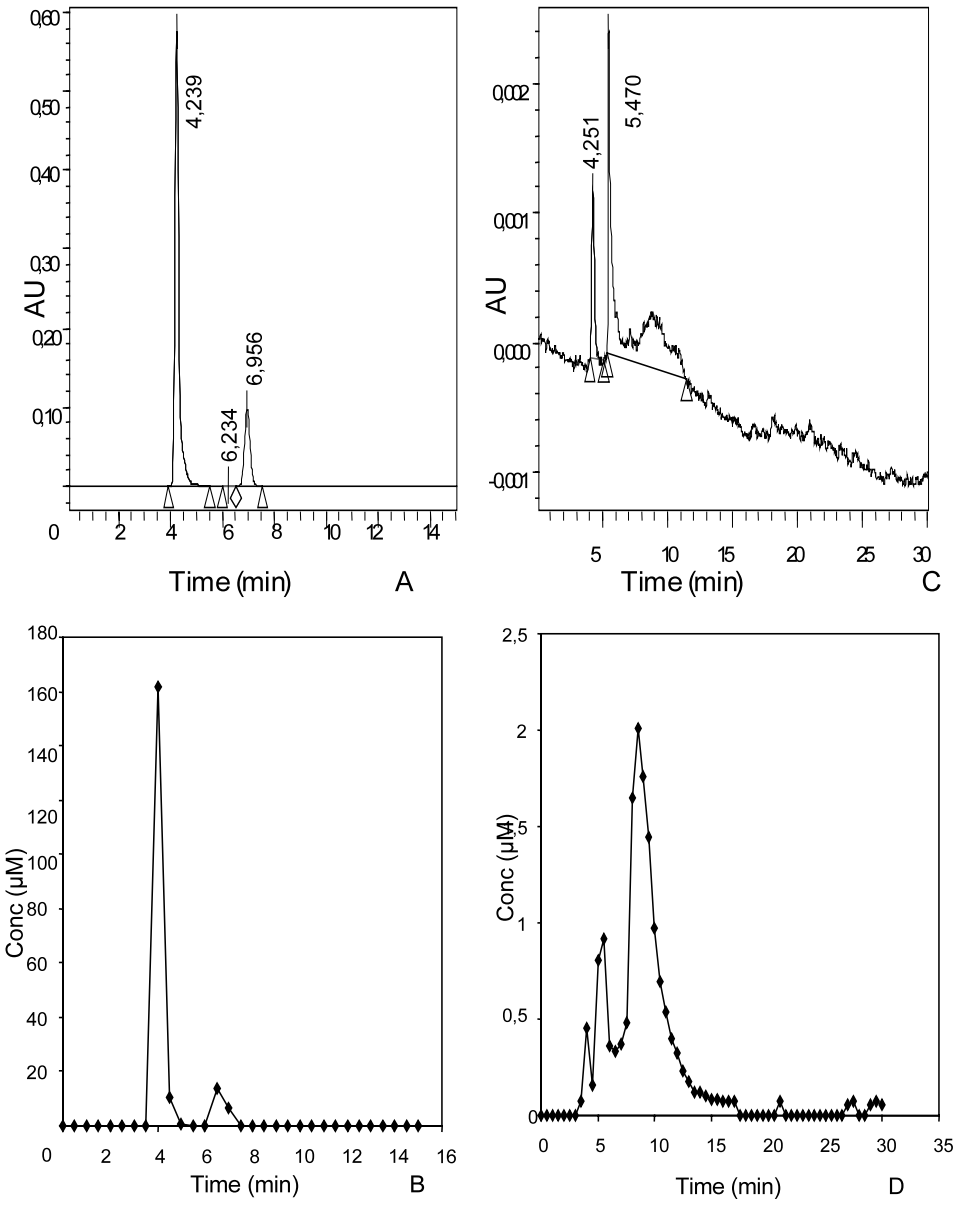

Fig. 7. A. HPLC chromatogram for NAMI-A at pH 7.4 immediately after preparation. B. F-AAS ruthenium concentration profiles for NAMI-A at pH 7.4 immediately after preparation. C. HPLC chromatogram for NAMI-A at pH 7.4 after 24 h storage. D. F-AAS ruthenium concentration profiles for NAMI-A at pH 7.4 after 24 h storage.

presence of a ruthenium-containing degradation product that is not fully detected using the HPLC method with detection at 358 nm.

From the F-AAS experiments, it can be concluded that under normal experimental conditions, all ruthenium injected into the HPLC system is recovered and all ruthenium-containing compounds form peaks in the chromatogram. However, for severely degraded solutions of NAMI-A some ruthenium is retained on the column and not all ruthenium-containing compounds are detected with the chromatographic system.

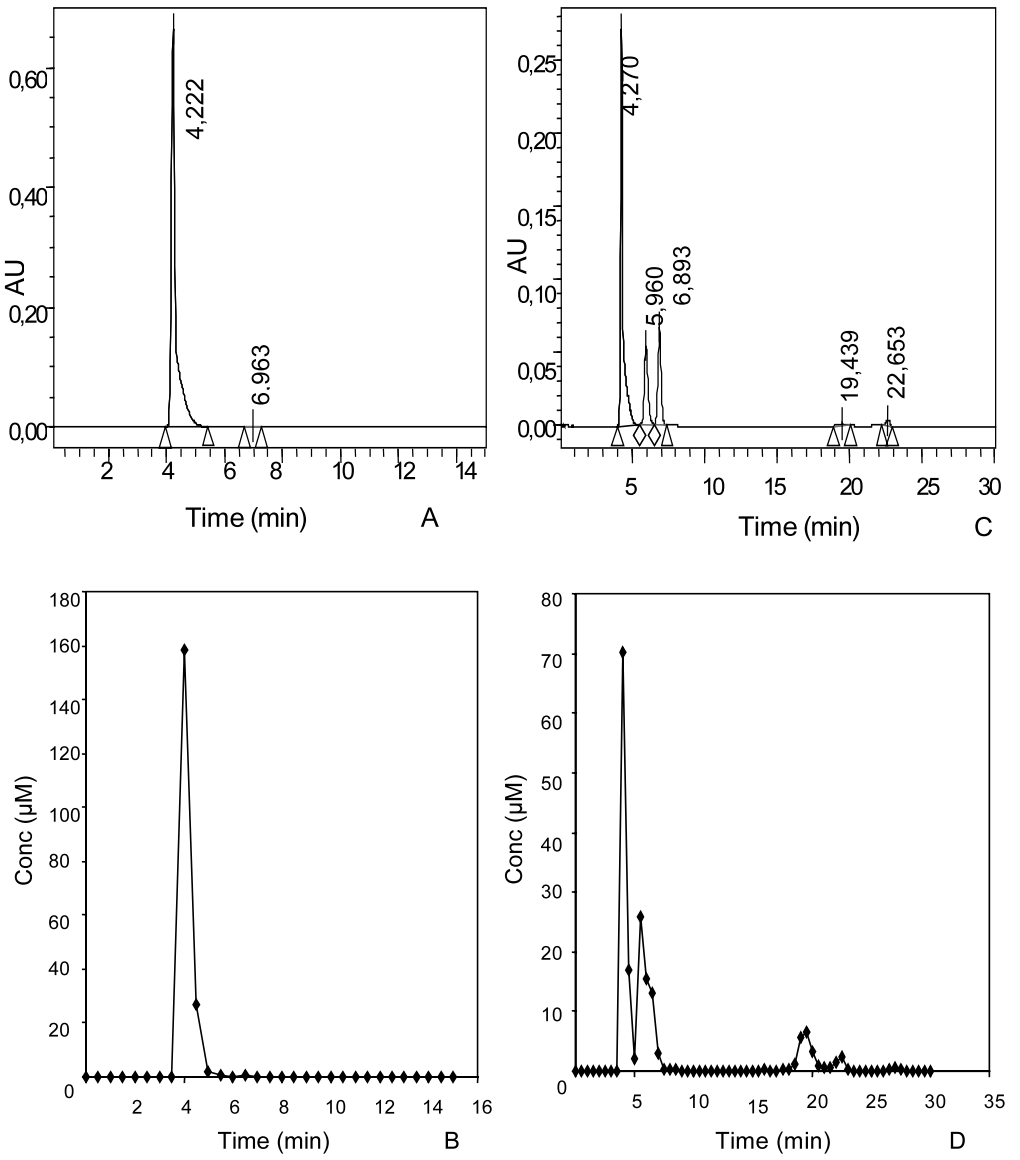

Fig. 8. A. HPLC chromatogram for NAMI-A at pH 3.5 immediately after preparation. B. F-AAS ruthenium concentration profiles for NAMI-A at pH 3.5 immediately after preparation. C. HPLC chromatogram for NAMI-A at pH 3.5 after 72 h storage. D. F-AAS ruthenium concentration profiles for NAMI-A at pH 3.5 after 72 h storage.

3.6. NAMI-A lyophilized product

NAMI-A lyophilized product shows an identical chromatogram to NAMI-A drug substance, with the main peak exhibiting NAMI-A's UV/VIS spectrum. The purity of the lyophilized product varies slightly with each manufactured batch, but is generally around 97%. The presence of one "impurity" is observed: the mono-hydroxy species of NAMI-A (compound B in Fig. 4). This is probably the result of degradation during the dissolution, filtration, and filling steps in the manufacturing process. The lyophilization process, however, does not affect the integrity of NAMI-A.

4. Conclusion

UV/VIS spectrophotometry and HPLC were used to analytically characterize NAMI-A. The HPLC method was validated and found to be linear, accurate, precise and stability indicating. Stress testing showed that degradation products were separated from the parent compound. One degradation product was identified as the mono-hydroxy product of NAMI-A. F-AAS experiments revealed that generally, all ruthenium that is injected onto the HPLC column is recovered and that all ruthenium-containing compounds produce a peak in the chromatogram. Upon severe degradation in alkaline solution, loss of NAMI-A and degradation products takes place as a result of precipitation and some ruthenium is retained on the column. However, for the pharmaceutical quality control of NAMI-A, a specification will be included to inject samples immediately after their preparation, minimizing degradation of the samples and thus circumventing detection and retention problems encountered with extremely degraded solutions of NAMI-A. Minimization of degradation of the samples in the autosampler vials is also achieved by diluting the samples with mobile phase, which has a pH of 2.5. An acidic pH has been shown to stabilize NAMI-A in solution.

NAMI-A lyophilized product shows identical chromatograms and UV/VIS spectra as NAMI-A drug substance. This stability-indicating HPLC method will be used in future analyses and pharmaceutical quality control of NAMI-A drug substance and lyophilized product.

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